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Tyrosinase as an autoantigen in patients with vitiligo

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SUMMARY

Vitiligo is considered an autoimmune disorder due to the generation and presence of autoantibodies directed against melanocyte antigens in the patients' sera. In the present study we point towards a newly defined autoantigen in vitiligo, the enzyme tyrosinase, which participates in the process of melanogenesis. Anti-tyrosinase antibodies were detected in the sera of seven patients with diffuse and 11 patients with localized vitiligo. Employing solid-phase ELISA to mushroom tyrosinase, we found that patients with diffuse vitiligo had significantly higher titres of IgG anti-tyrosinase autoantibodies than patients with localized disease or healthy subjects. These anti-tyrosinase autoantibodies have relatively high functional affinity to tyrosinase and can be recovered from vitiligo patients' sera by affinity purification. The anti-tyrosinase antibodies do not cross-react with other enzymes recognized as autoantigens in different autoimmune disorders and the autoantibodies do not block the enzymatic activity of tyrosinase, indicating that they are not reacting with the catalytic site of the enzyme. These data point to tyrosinase as an autoantigen in vitiligo and suggest that anti-tyrosinase titres can serve as a marker for disease activity.

Keywords vitiligo tyrosinase autoimmunity autoantibodies

INTRODUCTION

Vitiligo is a dermatologic disorder characterized by localized or diffuse depigmented patches on the skin [1,2]. Vitiligo is considered an autoimmune disease due to the following features: the presence of autoantibodies against melanocytes in patients' sera [3–7], the association of vitiligo with other autoimmune conditions [8–10], the presence of organ-specific autoantibodies in the patients' sera [11,12], the detection of autoantibodies in first-degree relatives of subjects with vitiligo [13], and the association of the disease with HLA-DR4 or HLA-DR1 [14–16]. The titres of antibodies against melanocytes in the sera of subjects with vitiligo correlate with the activity and extent of the disease. Only 50% of subjects with localized vitiligo were found to have antibodies against melanocytes in their sera in comparison with 93% of patients with the diffuse disease [17].

We have shown recently that anti-melanocyte antibodies derived from patients with diffuse vitiligo, bound to melanoma cell lines, inhibited their proliferative capacity, lysed the cells in the presence of complement, and significantly reduced the development of murine melanoma metastases *in vivo* [18]. In another study we demonstrated that antibodies directed against epitopes on melanoma cells (anti-B-16 antibodies) are present in higher titres in patients with vitiligo than in healthy volunteers and patients with malignant melanoma [19].

There is evidence that the anti-melanocyte antibodies in vitiligo have different target antigens on the surface cell membrane; some antigens are unique to pigmented cells, while others are expressed also on non-pigmented cells [20,21]. The question arises, whether the anti-melanocyte autoantibodies are pathogenic inducing vitiligo, or whether they are merely an epiphenomenon, e.g. reflecting an immunological response to the damaged melanocytes caused by other mechanisms [22].

Tyrosinase is a 75-kD copper-containing enzyme present only in cells that originate from the embryonal neural crest and is the key enzyme for the metabolism of melanin in pigmented cells and catecholamines in the neuroendocrine systems. Two types of tyrosinase enzymes have been isolated from melanocytes. Both are expressed in pigmented tissues [23–25] and show close homology of their amino acid sequences. The abundant type of enzyme is an intracellular membrane-bound enzyme, whereas the other exhibits soluble form [26]. Several possibilities were put forward to explain how tyrosinase can be exposed to the immunological system. The membrane soluble forms of tyrosinase from malignant or normal pigmented cells can serve as a target for the

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production of autoantibodies. Melanoma cells in culture release tyrosinase to the growth medium [27], and it was reported that tyrosinase activity can be detected in sera of patients with metastatic melanoma [28–30]. Moreover, melanocytes have phagocytic capacity and express MHC class II molecules [31] and thus can serve as antigen-presenting cells.

In the current study we elucidated the role of tyrosinase (derived from mushroom) as an autoantigen in vitiligo. Highly specific anti-tyrosinase antibodies were detected and did not cross-react with other enzymes found or reported to be autoantigens in other autoimmune diseases.

PATIENTS AND METHODS

Patients and controls

Sera samples from 18 patients with vitiligo (diffuse, $n = 7$, localized, $n = 11$) and 41 with malignant melanoma were assayed. Five of those with malignant melanoma had melanoma-associated hypopigmentation (MAH). Normal controls consisted of 12 healthy subjects. An additional 38 healthy blood donors were assayed only for IgG class anti-tyrosinase activity.

ELISA for anti-tyrosinase antibodies

Microtitre 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 100 μl of mushroom tyrosinase (Sigma, St Louis, MO) diluted to 50 μg/ml in 0·5 N bicarbonate buffer pH 9·6. Since recombinant human tyrosinase is not commercially available, we used mushroom tyrosinase, which has 16·32% identity and 41·08% similarity with human tyrosinase. After an overnight incubation at 4°C, plates were washed three times with PBS containing 0·05% Tween-20 (PBS–T) and once with PBS. The plates were then blocked with 0·5% gelatin in PBS for 1 h at room temperature and washed four times with PBS–T/PBS. The tested sera, diluted in 100 μl PBS, were applied for 1 h at 37°C. The plates were washed again, and alkaline phosphatase-conjugated $F(ab')_2$ anti-human IgG, IgA or IgM (Zymed, San Francisco, CA), diluted 1 : 1000 in PBS, was added for 1 h at 37°C. *p*-nitrophenylphosphate dissolved in a $NaHCO_3$ buffer containing $MgCl_2$ was added as a substrate for the alkaline phosphatase. Absorption was read at 405 nm on a Titertek multiscan microplate reader (Titertrac S.L.T. Laboratory Instruments, Vienna, Austria).

Functional affinity of the anti-tyrosinase antibodies

To measure the affinity between the anti-tyrosinase antibody and its target antigen, the method of Devey *et al*. [33,34] was used. Briefly, diethylamine (DEA) interferes with the binding of the antibody to the antigen. To find the optimal concentration of DEA, different concentrations (0·1–100 mM) were incubated with sera of patients with vitiligo in a fixed dilution of 1 : 50. From this calibration curve, the concentration of 10 mM DEA was found to represent the linear decline of the curve. Sera samples were serially diluted on ELISA plates coated with tyrosinase, in the presence or absence of 10 mM DEA (Merck, Münich, Germany). These plates were incubated at 37°C for 1 h and washed four times with PBS–T/PBS. Alkaline phosphatase-conjugated $F(ab')_2$ anti-human IgG was used. Dose–response curves were plotted and the decrease in log titre due to DEA was measured at half maximal readings (inhibition index). The extent of this fall in titre (which is expressed by the leftward displacement of the binding curve) was defined as an estimate of functional affinity (FA).

ELISA for other enzyme autoantigens

To assess the specificity of the serum to tyrosinase, ELISA plates were coated with the following enzyme autoantigens: myeloperoxidase (MPO), the autoantigen in vasculitis; pyruvate dehydrogenase (PDH), the autoantigen in primary biliary cirrhosis, neutrophil α-fraction containing proteinase-3 which is the autoantigen in Wegener's granulomatosis; protein extracted from kidney epithilial cell basal membrane, serving as an autoantigen in patients with Goodpasture's syndrome designated as NC-1 [35]. The antigens were diluted in the following concentrations: 1 mg/ml, 0·5 mg/ml, 0·1 mg/ml and 0·01 mg/ml in bicarbonate buffer, incubated overnight at 4°C and washed with PBS–T. The assayed sera diluted at 1 : 50 were incubated for 1 h at room temperature, washed, and the plates were processed as described above.

Tyrosinase enzymatic activity

The enzymatic activity of tyrosinase was determined according to the manufacturer's protocol (Sigma). The substrate catechol 0·17 mM with ascorbic acid 0·07 mM and EDTA 0·0022 mM were dissolved in a potassium phosphate buffer 50 mM pH 6·5 at 23°C, and then 40 U of tyrosinase were added to a final concentration of 3 ml. The decrease in optical density (OD) of the solution was read by spectrophotometer at a wavelength of 256 nm. After determining the linear slope of the enzymatic activity curve, sera of vitiligo and control patients diluted 1 : 50 were preincubated with the tyrosinase solution for 10 min at 23°C, and assayed for tyrosinase enzymatic activity.

Affinity purification of anti-tyrosinase antibodies

Cyanogen bromide-activated Sepharose 4B (Sigma) was rehydrated with 1 mM bicarbonate buffer for 2 h, and the free sites blocked by 1 h incubation with 0·1 M HCl for 15 min and extensively washed. Beads (600 mg) were gently stirred with 10 mg of tyrosinase dissolved in 0·1 mM bicarbonate buffer for 2 h, and the free sites blocked by 1 h incubation with 0·1 M glycine. The beads were then centrifuged at 400 ***g*** for 5 min, and washed three times with 0·1 M Na-acetate pH 4·0, supplemented with 0·5 M NaCl. Sera were passed over the column and left at 4°C for 3 h. Anti-tyrosinase antibodies were then eluted with 0·2 M glycine–HCl pH 2·8, neutralized immediately with NaOH, suspended in the original volume in PBS, and examined as above.

Other serological tests

Anti-double stranded (ds) DNA antibodies were evaluated using an in-house assay. Commercial kits were used to detect anti-Sm, anti-U1RNP, anti-Ro/SSA and anti-La/SSB antibodies. IgM, IgG and IgA rheumatoid factor (RF) activity was determined by ELISA (Eldan Tech, Jerusalem, Israel).

Statistical analysis

The figures quoted below are arithmetic means and s.d. The cut-off level for all tests was set at 3 s.d. above the mean value of the normal control. Statistical analysis included descriptive statistics and Wilcoxon's test for paired data.

RESULTS

The anti-tyrosinase IgG antibody titres are shown in Fig. 1, indicating (at a dilution of 1 : 50) that the sera of patients with diffuse vitiligo (mean OD $= 0{\cdot}658 \pm 0{\cdot}251$) contain significantly

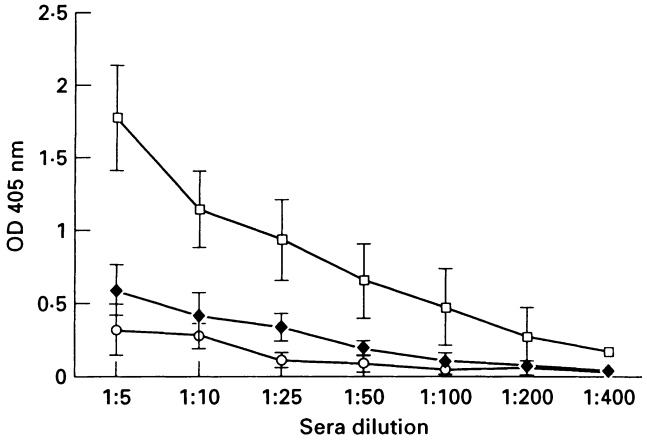

Fig. 1. Sera dilution curve of anti-tyrosinase antibodies measured by ELISA. Sera of patients with diffuse vitiligo (□) have high significant anti-tyrosinase antibodies up to 1 : 100 dilution ($P = 0{\cdot}003$), whereas sera of patients with localized vitiligo (◆) show low non-significant anti-tyrosinase antibody titre in comparison with normal healthy controls (○).

higher levels of the IgG isotype anti-tyrosinase antibodies than sera from patients with localized vitiligo (mean OD = 0·199 ± 0·05) and from the group of healthy subjects (mean OD = 0·093± 0·049). Sera from patients with localized disease showed non-significantly lower titres of anti-tyrosinase antibodies in comparison with apparently healthy controls. The isotype distribution of the anti-tyrosinase antibodies is depicted in Fig. 2, emphasizing that most anti-tyrosinase reactivity is due to IgG (61·0%) in patients with vitiligo, 58·3% in those with malignant melanoma, and 60·0% in those with malignant melanoma associated with vitiligo. Four patients with melanoma had only IgA anti-tyrosinase and one melanoma patient had only IgM antibodies.

Anti-tyrosinase antibodies' affinity was measured in the sera of six patients with vitiligo who showed the highest IgG anti-tyrosinase titres. Figure 3 depicts the high functional affinity of these sera, which is expressed by a relatively small leftward

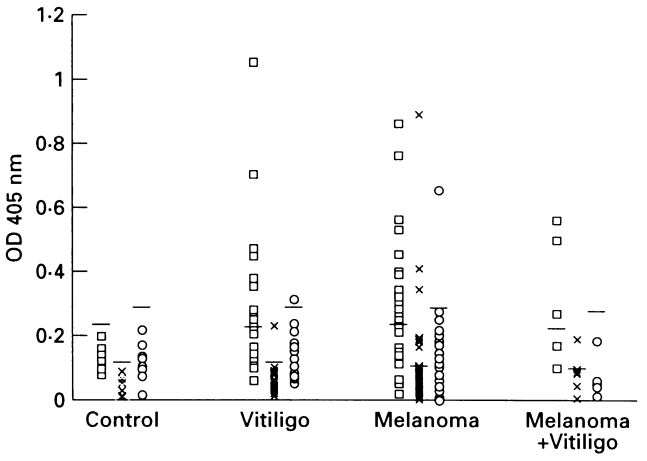

Fig. 2. The isotope distribution of anti-tyrosinase antibodies in vitiligo, melanoma and melanoma patients who developed vitiligo. The horizontal bars signify the cut-off points defining the positive titres, calculated as the mean + 3 s.d. of the normal sera (IgG (□) = 0·240, IgA (×) = 0·130, IgM (○) = 0·304).

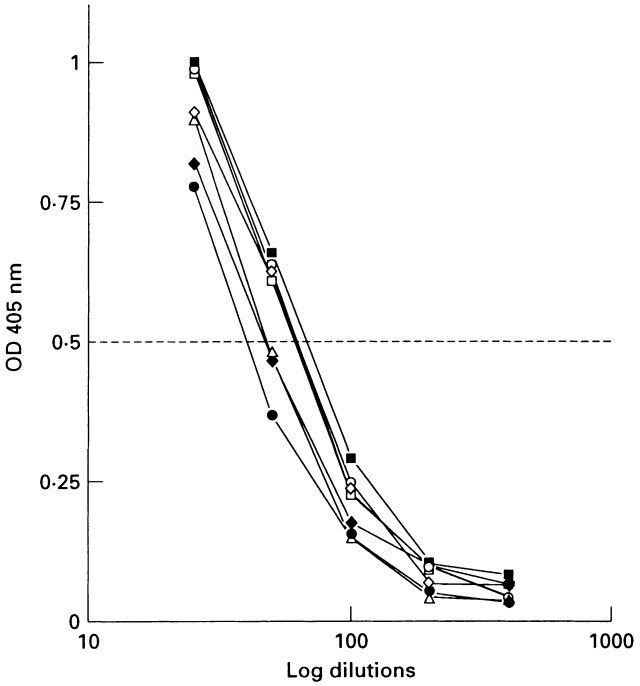

Fig 3. The functional affinity measurements of anti-tyrosinase antibodies in six vitiligo patients that showed the highest antibody titres in the ELISA. The small leftward displacement of the sera by dilution curves treated with diethylamine (DEA) in relation to untreated sera (measured at 50% of the maximal reading) reflects the high functional affinity of the IgG anti-tyrosinase antibodies to their antigen. ■, No DEA; ◇, vitiligo no. 1 + DEA; ○, vitiligo no. 2 + DEA; △, vitiligo no. 3 + DEA; □, vitiligo no. 4 + DEA; ◆, vitiligo no. 5 + DEA; ●, vitiligo no. 6 + DEA.

displacement from the serum without DEA that serves as a control. To exemplify the specificity of the anti-tyrosinase antibodies, the antibodies were further purified on an affinity column, and were almost completely (mean 83·1%) eluted from the sera by affinity purification (Table 1).

We could not determine cross-reactivity between the anti-tyrosinase antibodies in sera of patients with vitiligo and other enzyme autoantigens tested: MPO, proteinase-3, PDH, and the irrelevant protein autoantigen NC-1 (Fig. 4). The serum of patients with vitiligo and/or malignant melanoma had no anti-dsDNA, anti-Sm, anti-U1-RNP, anti-Ro/SSA or anti-La/SSB activity, excluding a concomitant serological marker of other systemic autoimmune disorders in these patients. Studying the condition of the enzymatic

Table 1. Affinity purification of anti-tyrosinase antibodies from vitiligo patients' sera detected by ELISA

Vitiligo	Eluate	Effluent	Recovery (%)
1	0·752	0·044	95
2	0·852	0·076	85
3	0·341	0·072	82
4	0·341	0·072	72
5	0·360	0·141	79
6	0·472	0·079	86

activity of tyrosinase in the presence of serum from vitiligo patients or normal controls revealed no effect on the catalytic process (data not shown).

DISCUSSION

The presence of autoantibodies directed against pigmented cells in sera of patients with vitiligo is well established, and there is a direct correlation between the level of these autoantibodies and disease activity [17,36]. The present study describes additional autoantigen in vitiligo, namely the enzyme tyrosinase. Employing solid-phase ELISA with the commercial mushroom tyrosinase, we found that sera of most patients with diffuse vitiligo contain significantly high titres of anti-tyrosinase antibodies exhibiting high affinity, with no cross-reactivity to other enzymes, while vitiligo patients with localized disease have non-significantly low titres of anti-tyrosinase antibodies. These findings are in line with those reported by Song *et al*. [21]. The presence of autoantibodies directed to the melanocytes' relatively specific enzyme is an additional support for the assumption that vitiligo has an autoimmune etiology.

Human melanoma cells excrete tyrosinase to the medium *in vitro* [37]. Tyrosinase was found in the sera of melanoma patients but not in sera of patients with other malignancies [38]. The production of anti-tyrosinase antibodies which was reported in malignant melanoma [39,40] and the finding that the tyrosinase gene codes for an antigen recognized by cytotoxic T cells in melanoma [41], both indicate that the immune system can react against tyrosinase in pathological circumstances. Moreover, the melanocyte itself has phagocytic capacity and can function as antigen presenting by expressing MHC class II [32]. These data indicate that exposure and presentation of tyrosinase to the immune system can occur, but the question regarding the breakage of self-tolerance leading to the emergence of autoantibodies directed to tyrosinase and the establishment of the autoimmune disorder vitiligo remains unanswered.

The mushroom tyrosinase is a tetrameric glycoprotein copper containing metalloenzyme with a molecular weight of $128 \pm 6{\cdot}4$ kD and a subunit mol. wt estimated at 32 kD [42]. The enzyme catalyses the first two reactions in the synthesis of the pigment melanin. In mammalian cells it has a soluble and a membranous form [43].

Enzymes are known to be autoantigens in various autoimmune disorders [35], thus the presence of anti-tyrosinase antibodies in vitiligo supports the hypothesis that vitiligo is a member of this group of diseases. There were no cross-reactions among tyrosinase, PDH, MPO and the α-fraction, e.g. proteinase 3, indicating that anti-tyrosinase antibodies are specific to tyrosinase. Moreover, affinity-purified sera of patients with systemic autoimmune disorders (systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome, systemic scleroderma and vasculitis) had no anti-tyrosinase reactivity (data not shown), supporting the possibility that these antibodies are unique to vitiligo. The anti-tyrosinase antibodies did not inhibit tyrosinase enzymatic activity, thus it seems that these antibodies are not directed against the catalytic site.

Our results support the possibility that vitiligo is an autoimmune disease. The simple ELISA described may serve as a serological test for disease activity. The role of anti-tyrosinase autoantibodies in the pathogenesis of vitiligo merits further investigation.

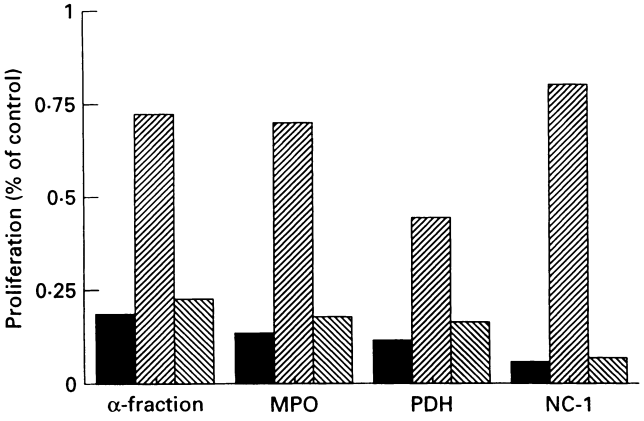

Fig. 4. The sera of vitiligo patients show similar non-significant binding to various enzyme autoantigens, as did normal human sera. Sera from patients with Wegener's granulomatosis show high titres for the α-fraction; sera from patients with vasculitis show high titres for myeloperoxidase (MPO); sera from patients with primary biliary cirrhosis show high titres for pyruvate dehydrogenase (PDH); sera from patients with Goodpasture's syndrome show high titres for protein extracted from kidney epithilial cell basal membrane NC-1. ■, Normal human serum control; ▨, positive control; ▧, vitiligo.

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REFERENCES

1 Nordlund JJ, Lerner AB. Vitiligo: it is important. (Editorial). Arch Dermatol 1982; **18**:5–8.
2 McBurney EL. Vitiligo: clinical picture and pathogenesis. Arch Intern Med 1979; **139**:1295–7.
3 Moellmann GE, Krass P, Halaban R. On the subject of serum antibodies to melanocytes. J Invest Dermatol 1985; **84**:333–4.
4 Galbraith GMP, Miller D, Emerson DL. Western blot analysis of serum antibody reactivity with human melanoma cell antigens in alopecia areata and vitiligo. Clin Immunopathol 1988; **48**:317–24.
5 Naughton G, Reggiardo D, Bystryn JC. Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. J Am Acad Dermatol 1986: **15**:978–81.
6 Naughton GK, Eisinger M, Bystryn JC. Antibodies to normal human melanocytes in vitiligo. J Exp Med 1983; **158**:246–51.
7 Hertz KC, Gazze LA, Charles AB, Kirkpatrick H, Katz SI. Autoimmune vitiligo-detection of antibodies to melanin producing cells. New Engl J Med 1977; **297**:634–7.
8 El Mofty A, El Morf M. Vitiligo: a symptom complex. Int J Dermatol 1980; **19**:237–44.
9 Macron C, Winter KI, Traisman HS. Vitiligo and juvenile diabetes melitus. Arch Dermatol 1977; **113**:1515–9.
10 Harzoulis P, Kanakoudi-Tsakalidis F, Uyzantiadis A. Autoimmunity and vitiligo (abstract). Arch Dermatol 1970; **114**:1554–60.
11 Bettere C, Del Prete GF, Preserico A, Bersani G, Caracciolo F, Trisotto A, Poggy F. Autoantibodies in vitiligo. Arch Dermatol 1976; **112**:1328.
12 Brostoff J, Bor S, Feiwel M. Autoantibodies in patients with vitiligo. Lancet 1969; **2**:177.
13 Grimes PF, Halder R, Jones C. Autoantibodies and their clinical significance in black vitiligo population. Arch Dermatol 1983; **119**:300–3.
14 Dunston GM, Halder RM. Vitiligo is associated with HLA-DR4 in black patients: a preliminary report. Arch Dermatol 1990; **126**:56–60.
15 Poloy A, Tibor L, Kramer J, Anh-Tuan N, Kraszite E. HLA-DR1 is associated with vitiligo. Immunol Letters 1991; **27**:59–62.

16 Foley LM, Lowe NJ, Mischelhoff E, Tiwari JL. Association of HLA-DR4 with vitiligo. J Am Acad Dermatol 1983; **8**:39–42.
17 Naughton G, Reggiardo D, Bystryn JC. Correlation between vitiligo antibodies and extent of depigmentation in vitiligo. J Am Acad Dermatol 1989; **15**:978.
18 Fishman P, Azizi E, Shoenfeld Y *et al*. Vitiligo antibodies are effective against melanoma. Cancer 1993; **72**:8–15.
19 Merimsky O, Shoenfeld Y, Yecheskel G, Chaichik S, Azizi E, Fishman P. Vitiligo and melanoma associated hypopigmentation: a similar appearance but different mechanisms. Cancer Immunol 1994; **38**: 411–6.
20 Cui J, Harning R, Henn M, Bystryn JC. Identification of pigment cell antigen defined by vitiligo antibodies. J Invest Dermatol 1992; **98**: 162–5.
21 Song YH, Connor E, Li Y, Zorovich B, Maclaren N. The role of tyrosinase in autoimmune vitiligo. Lancet 1994; **344**:1049–52.
22 Norris DA, Kissinger RM, Naughton GM, Bystryn JC. Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocytes *in vitro* by complement-mediated damage and antibody-dependent cellular cytotoxicity. J Invest Dermatol 1988; **90**:783–9.
23 Shibahara S, Tomita Y, Sakahura T, Nagar C, Chaudhuri B, Muller R. Cloning and expression of DNA encoding mouse tyrosinase. Nucleic Acids Res 1986; **14**:2413–27.
24 Jimenez M, Lee Maloy W, Hearing VJ. Specific identification of an authentic clone for mammalian tyrosinase. J Biol Chem 1989; **264**: 3397–403.
25 Yamamato H, Takeuchi S, Kudo T, Sato C, Takeuchi T. Melanin production in culture albino melanocytes transfected with mouse tyrosinase cDNA. Jap J Genet 1989; **64**:121–35.
26 Wittbjer A, Odh G, Rosengren AM, Rorsman H. Isolation of soluble tyrosinase from human melanoma. Acta Derm Venereol (Stokholm) 1990; **70**:291–4.
27 Karg E, Hultberg B, Isaksson A, Rosengren E, Rorsman H. Enzyme release from cultured human melanoma cells. Acta Derm Venereol (Stokholm) 1990; **70**:286–90.
28 Sohn N, Gang H, Gumport SL, Goldstein M, Depisch LM. Generalized melanosis secondary to malignant melanoma. Cancer 1969; **24**:897–903.
29 Nishioka K, Romsdahl MM, McMurtrey MJ. Adaptation of triatiated tyrosinase assay to serum tyrosinase and its specific elevation in melanoma. In: Klaus SN, ed. Pigmented cell 1979; **5**:300–4.
30 Agrup P, Carstam R, Wittbjer A, Rorsman H, Tosengren E. Tyrosinase activity in serum from patients with malignant melanoma. Acta Derm Venereon (Stokholm) 1989; **69**:120–4.
31 Al Badri AMT, Foulis AK, Todd PM, Garioch JJ, Gudgeon JE, Stewart DG, Gracie JA, Goudie RB. Abnormal expression of MHC class II and ICAM-1 by melanocytes in vitiligo. J Pathol 1993; **169**:203–6.
32 Le Poole IC, Mutis T, van den Wijngaard R, Westerhof W, Ottenhoff T, de Vries A, Das PK. A noval, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. J Immunol 1993; **151**:7284–92.
33 Devey ME, Bleasdale K, Lee S, Rath S. Determination of functional affinity of IgG1 and IgG4 antibodies to tetanus toxoid by isotype-specific solid-phase assay. J Immunol Methods 1988; **106**:119–25.
34 Youinou P, Keusch J, Casbrun-Budd R, Paolozzi L, Lamour A, Le Goff P, Bendauod B. Tiopronine induced reduction of rheumatoid factor functional affinity and asialylated IgG in patients with rheumatoid arthritis. Clin Exp Rheumatol 1992; **10**:333–8.
35 Tomer Y, Gilburd B, Blank M *et al*. Characterization of biologically active ANCA induced in mice: pathogenetic role in experimental vasculitis. Arthritis Rheum 1995; **38**:1375–81.
36 Harning R, Cui J, Bystryn JC. Relation between the incidence and level of pigment cell antibodies and disease activity in vitiligo. J Invest Dermatol 1991; **97**:1078–80.
37 Karg E, Jultberg B, Isakson A, Rosengren E, Rorsman H. Enzyme release from culture human melanoma cells. Acta Derm Venereol 1990; **70**:268–90.
38 Nishioka K, Romsdahl MM, McMurtery MJ. Adaptation of tritiated tyrosinase assay to serum tyrosinase and its specific elevation in melanoma. Pigment Cell 1979; **5**:300–4.
39 Bouchard B, Vijayasaradhi S, Houghton AN. Production and characterization of antibodies against human tyrosinase. J Invest Dermatol 1994; **102**:291–5.
40 Baharav E, Merinsky O, Shoenfeld Y, Altomonte M, Maio M, Ferrone S, Fishman P. Anti-tyrosinase antibodies participate in the immune response to vaccination with anti-idiotypic antibodies mimicking the high molecular weight melanoma associated antigen. Melanoma Research 1995; **5**:337–48.
41 Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plean E, Lethe' B, Coulie P, Boon T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocites on HLA-A2 melanoma. J Exp Med 1993; **178**:489–95.
42 Jolley RL Jr, Robb DA, Mason HS. The multiple forms of mushroom tyrosinase. J. Biol Chem 1969; **244**:1593–9.
43 Wittbjer A, Odh G, Rosengren AM, Rosengren E, Rorsman H. Isolation of soluble tyrosinase from human melanoma cells. Acta Derm Venereol 1990; **70**:291–4.